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Determining the Repertoire of *IGH* Gene Rearrangements to Develop Molecular Markers for Minimal Residual Disease in B-Lineage Acute Lymphoblastic Leukemia

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Molecular markers for minimal residual disease in B-lineage acute lymphoblastic leukemia were identified by determining, at the time of diagnosis, the repertoire of rearrangements of the immunoglobulin heavy chain (*IGH*) gene using segment-specific variable (V), diversity (D), and junctional (J) primers in two different studies that involved a total study population of 75 children and 18 adults. This strategy, termed repertoire analysis, was compared with the conventional strategy of identifying markers using family-specific V, D, and J primers for a variety of antigen receptor genes. Repertoire analysis detected significantly more markers for the major leukemic clone than did the conventional strategy, and one or more IgH rearrangements that were suitable for monitoring the major clone were detected in 96% of children and 94% of adults. Repertoire analysis also detected significantly more *IGH* markers for minor clones. Some minor clones were quite large and a proportion of them would not be able to be detected by a minimal residual disease test directed to the marker for the major clone. *IGH* repertoire analysis at diagnosis has potential advantages for the identification of molecular markers for the quantification of minimal residual disease in acute lymphoblastic leukemia cases. An *IGH* marker enables very sensitive quantification of the major leukemic clone, and the detection of minor clones may enable early identification of additional patients who are prone to relapse. ***(J Mol Diagn 2009, 11:194–200; DOI: 10.2353/jmoldx.2009.080047)***

The magnitude of the early response to chemotherapy is a powerful prognostic factor in acute lymphoblastic leukemia (ALL)[1–3] and, to assess this, rearrangements of the immunoglobulin and/or T-cell receptor genes are now widely used as molecular markers for measuring the level of minimal residual disease (MRD). The most common approach for identifying markers, used by many laboratories including the Sydney group, involves screening for a variety of immunoglobulin and T-cell receptor gene rearrangements by polymerase chain reaction (PCR) using family-specific primers for variable (V), diversity (D), and junctional (J) segments.[4–8] The Flinders group has developed a somewhat different approach, termed repertoire analysis, which uses primers directed against individual V, D, and J segments of the immunoglobulin heavy chain gene (*IGH*) to identify rearrangements present at diagnosis in B-lineage ALL. An initial study by the Flinders group showed that the repertoire strategy had some advantages and consequently a second study was performed in collaboration with the Sydney group to enable a direct comparison of the two approaches using the same set of patient samples. In this paper we report the results of both sets of studies, which illustrate the utility of repertoire analysis for marker detection and highlight the occurrence of clonal evolution in ALL.

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M.J.B., P.J.S., A.A.M., and Flinders University hold equity in Monoquant P/L, which has submitted a patent application to cover repertoire analysis.

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Table 1. Primer Sequences Used for Repertoire Analysis

V_H segment-specific primers			
V1.02	5′-ATCAACCCTAACAGTGGTGG-3′	V3.33	5′-AGTGGGTGGCAGTTATATGG-3′
V1.03	5′-GCTGGCAATGGTAACACAAAA-3′	V3.43	5′-GGTCTCTCTTATTAGTTGGGA-3′
V1.08	5′-ACCTAACAGTGGTAACACAGG-3′	V3.49	5′-ATGGTGGGACAACAGAATACA-3′
V1.18	5′-GGGATGGATCAGCGCTT-3′	V3.53	5′-GTGGGTCTCAGTTATTTATAGC-3′
V1.24	5′-TGGAGGTTTTGATCCTGAAGA-3′	V3.64	5′-CTCAGCTATTAGTAGTAATGGG-3′
V1.45	5′-ACACCTTTCAATGGTAACACC-3′	V3.72	5′-AAACAAAGCTAACAGTTACACC-3′
V1.46	5′-GGGAATAATCAACCCTAGTGG-3′	V3.73	5′-AAGCAAAGCTAACAGTTACG-3′
V1.58	5′-GATAGGATGGATCGTCGTTG-3′	V3.74	5′-TCACGTATTAATAGTGATGGGA-3′
V1.69	5′-TCATCCCTATCTTTGGTACAG-3′	V3D	5′-TCCATTAGTGGTGGTAGCA-3′
V2.05	5′-ACTCATTTATTGGAATGATGATAAG-3′	V4.04	5′-CCATCAGCAGTAGTAACTGG-3′
V2.26	5′-ACACATTTTTTCGAATGACGAA-3′	V4.28	5′-GCAGTAGTAACTGGTGGG-3′
V2.70	5′-TGATTGGGATGATGATAAATTCT-3′	V4.30.1	5′-GACTGGTGAAGCCTTCACA-3′
V3.07	5′-AGCAAGATGGAAGTGAGAAA-3′	V4.34	5′-ATGGTGGGTCCTTCAGTG-3′
V3.09	5′-GGAATAGTGGTAGCATAGGC-3′	V4.39	5′-AGAGTCGAGTCACCATATCC-3′
V3.11	5′-CATTAGTAGTAGTGGTAGTACCAT-3′	V4.59	5′-CTGGTGGCTCCATCAGTA-3′
V3.13	5′-TCTCAGCTATTGGTACTGC-3′	V4.61	5′-GTCTCTGGTGGCTCCG-3′
V3.15	5′-GCGGTATTAAAAGCAAAACTG-3′	V5.51	5′-CTGGTGACTCTGATACCAGA-3′
V3.20	5′-GCTGGAGTGGGTCTCT-3′	V5A	5′-ATCCTAGTGACTCTTATACCAAC-3′
V3.21	5′-CATCCATTAGTAGTAGTAGTAGTT-3′	V6.01	5′-CATACTACAGGTCCAAGTGG-3′
V3.23	5′-GTGGGTCTCAGCTATTAGTG-3′	V7.4.1	5′-GATCAACACCAACACTGGG-3′
V3.30	5′-AGTGGGTGGCAGTTATATCA-3′		
6 D_H primers for D-J partial rearrangements			
D1 out	5′-ACCCAGGAGGCCCCAGAGCTCAGGG-3′	D1 in	5′-CCCGGTCGGATTCTGAACAGCCCCGA-3′
D2 out	5′-CACCMGKAGGGACAGGAGGATTTTGTGGG GG-3′	D2 in	5′-GGAGGATTTTGTGGGGGCTCGTGTCACTG-3′
D3 out	5′-GCCCTGGACATCCCGGGTTTCCCCAGG-3′	D3 in	5′-GGGTTTCCCCAGGCCTGGCGGTAGGTTT-3′
D4 out	5′-TGGACCAGGGCCTGCGTGGGAAAGG-3′	D4 in	5′-GCGTGGGAAAGGCCTCTGGSCACACTC-3′
D5 out	5′-GCCCCGCCTCCAGTTCCAGGTGTGG-3′	D5 in	5′-GCCTCCAGTTCCAGGTGTGGTTATTGTCA GG-3′
D6 out	5′-GNGGKGCTGAGCCCAGCAAGGGAAGG-3′	D6 in	5′-GCCCAGCAAGGGAAGGCCCCCAAACA-3′
D7 out	5′-CAGGCCCCCTACCAGCCGCAGGG-3′	D7 in	5′-AGCCGCAGGGTTTTGGCTGAGCTG-3′
J_H specific primers (from the intron between J segments)			
J1a	5′-TCCCCAAGTCTGAAGCCA-3′	J4a	5′-TCCGGGGCTCTCTTGG-3′
J1b	5′-CGACCTCCTTTGCTGAG-3′	J4b	5′-TTGCCCCTCGTCTGTGT-3′
J2a	5′-GGAGGGGGCTGCAGTG-3′	J5a	5′-GCAAGCTGAGTCTCCCT-3′
J2b	5′-GGCTGCAGACCCCAGA-3′	J5b	5′-CTTTCTTTCCTGACCTCCAA-3′
J3a	5′-CCCAGCTCCAGGACAGA-3′	J6a	5′-ACAAAGGCCCTAGAGTGG-3′
J3b	5′-CAGCGCAGACCAAGGA-3′	J6b	5′-CCCACAGGCAGTAGCAG-3′
Set of 7 V_H primers for multiplexing for pre-amplification			
IgH pre1	5′-GGCTCTGGCAGGTGCAGCTGGTGGAGTCTGG-3′	IgH pre5	5′-GGCTCTGGCAGGTGCAGCTGGTGGAGTCCGG-3′
IgH pre2	5′-GGCTCTGGCAGGTGCAGCTGGTGCAGTCTGG-3′	IgH pre6	5′-GGCTCTGGCAGGTACAGCTGCAGCAGTCAGG-3′
IgH pre3	5′-GGCTCTGGCAGGTCACCTTGAAGGAGTCTGG-3′	IgH pre7	5′-GGCTCTGGCAGGTGCAGCTACAGCAGTGGGG-3′
IgH pre4	5′-GGCTCTGGCAGGTGCAGCTGCAGGAGTCGGG-3′		
Consensus primers			
FR3A (in V)	5′-ACACGGCCGTGTATTACTGT-3′	VLJH (in J)	5′-GTGACCAGGGTNCCTTGGCCCCAG-3′
FR2B (in V)	5′-GTCCTGCAGGCYYCCGGRAARRGTCTGGAGTGG-3′	ELJH (in J)	5′-TGAGGAGACGGTGACCAGGATCCCTTGGCCCCAG-3′
TaqMan probes in J			
J probe1	5′-TCACCGTCTCCTCAGG-3′	J probe2	5′-TCACTGTCTCCTCAGG-3′

Materials and Methods

The first study used bone marrow samples obtained at diagnosis from 25 children and 18 adults with B-lineage ALL. Ethical approval was obtained for the procurement of all samples. Genomic DNA was extracted using the Qiagen Flexigene DNA kit according to the manufacturer's instructions.

The second study used bone marrow samples obtained from another 50 children with B-lineage ALL. The patients came from a series of 50 consecutive patients with B-lineage ALL treated at Sydney Children's Hospital in the Australian and New Zealand Children's Hematology Oncology Group (ANZCHOG) Study 8 clinical trial. DNA was extracted from Ficoll purified mononuclear cells using Machery-Nagel Nucleobond column purification according to the manufacturer's instructions.

The detailed methods below are those used for repertoire analysis in the Flinders laboratory. The methods used for conventional analysis in the Sydney laboratory are described subsequently.

PCR

Unless otherwise stated, duplicate amplifications were performed, each in a volume of 25 μl and containing 2 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 4 mmol/L $MgCl_2$, 200 μmol/L each of dATP, dUTP, dCTP, and dGTP, 100 ng of each primer, 1 unit of Platinum Taq (Invitrogen) and, for Q-PCR, 4 pmol of a TaqMan probe to a conserved sequence in the J segment. Cycling conditions were 92°C for 15 seconds followed by 58°C for 1 minute and 72°C for 15 seconds. Unless otherwise stated, the mass of genomic DNA in each PCR was 2 ng in the first study and 20 ng in the second study.

Primers and Probes

The sequences of all primers and probes used for *IGH* repertoire analysis are shown in Table 1. The primers were designed to cover as comprehensively as possible the 52 functional V_H regions listed in the IMGT database.[9]

Thus, 32 primers were designed to match just one V_H segment; eight primers to match two V_H segments each; and one primer to match four homologous V_H4 segments. Two TaqMan probes were used, the J probe1 for J1 and J3–J6 and J probe2 for the J2 segment. It should be noted that the consensus primer FR2B amplifies Vh1 family segments relatively inefficiently.

Preamplification of Genomic DNA

Preamplification performed two functions. It enabled study of a relatively large amount of genomic DNA to minimize sampling error and facilitate detection of low-abundance rearrangements, and it provided sufficient material for the large number of PCRs used in subsequent analysis. Samples from 10 children and 10 adults in the first study and the 50 patients in the collaborative study were preamplified using *IGH* generic primers. Two or three replicate samples, each of 50 ng of genomic DNA, were preamplified for 15 cycles in a multiplex PCR that included 20 ng each of seven V primers (IGH pre 1–7) designed to cover all framework 1 sequences in the germline V_H regions and 20 ng each of six J primers (1a, 2a, 3b, 4a, 5a, 6a) designed to cover all J sequences of the *IGH* locus.

Identification of V, D, and J Segment Usage

J Segment Usage

Consensus forward primers FR3A and FR2B and/or the pool of seven D_{OUT} segment primers were tested against primers (both Ja and Jb) for each of the six individual J segments by Q-PCR using 100 ng of each primer, 20 ng of genomic DNA, and both the J1 and J2 probes. The J segment involved in a rearrangement was identified from the cycle threshold (Ct) value together with visualization of an appropriately sized band after electrophoresis.

V Segment Usage

V segments were identified after determining J segment usage. In the first study, V segment usage was determined using genomic DNA, although in 10 children and 10 adults it was also determined using preamplified DNA. In the second study, a 1:1000 dilution of preamplified DNA was used to identify candidate V segments, which were confirmed using 50 ng of genomic DNA.

V segment identification involved separate PCR testing of 41 forward V_H segment primers against the previously identified reverse J primer. Where a V-specific primer gave a Ct within 12 cycles of that given by the control primers, FR3A and FR2B, electrophoresis was used to confirm the presence of a band of appropriate size, in the range of 200 to 500 bp.

D Segment Usage

In the first study, screening for DJ rearrangements was only performed if a complete VDJ rearrangement had not been identified; however, in the collaborative study, all samples were screened for DJ rearrangements. Incomplete D-J rearrangements were identified by Q-PCR on genomic DNA with a mixture of the seven forward D_{OUT} segment primers tested with the six individual reverse J primers, followed by testing individual D_{IN} primers with the previously identified J primer. Amplification of a DJ rearrangement gave a sharp gel band in the range of 90 to 300 bp.

Sequencing

Candidate rearrangements amplified using specific V-J or D-J primer pairs were cut from gels and sequenced using an ABI 3100 genetic analyzer. To confirm the identity of V, D, and J regions used, sequences were screened using the IMGT database[9] and V-QUEST tool.[10] Rearrangements were assumed to belong to the same lineage if they appeared to have been created by V region replacement, ie, they shared the same J region, N-J junction and N region, and possibly part of the D region as well. In assigning the order in which clones arose, it was assumed that in V region replacement, a downstream V region is replaced by another V segment further upstream. A rearrangement was assumed to belong to a leukemic clone either if it had been seen in two amplifications that had originated from two separate aliquots of genomic DNA or if the sequence indicated a lineage relationship to that of a leukemic clone already identified.

Abundance of Rearrangements and Assignment of Markers to Major or Minor Clones

All rearrangements detected in a sample were amplified from genomic DNA in the one experiment, and the relative abundance of each was calculated using the observed Ct value and the figure of 1.96, as determined experimentally in our laboratory for the amplification factor per PCR cycle. Since estimates of abundance are not precise, we used the following broad criteria to assign a marker to the major, ie, dominant clone or to a minor clone. If there was only one rearrangement that had an abundance of $>10\%$, and its abundance was at least 10-fold greater than that of the next most abundant rearrangement, then this rearrangement was regarded as marking a major clone in which the cells exhibited a monoallelic rearrangement. If there were two or more rearrangements, each with an abundance of $>10\%$, then the two most abundant were regarded as marking the major clone in which the cells exhibited a biallelic rearrangement. All other rearrangements, irrespective of their abundance, were regarded as marking minor clones.

Comparison with the BIOMED-2 Approach for Detection of Rearrangements

In the first study, detection of complete *IGH* rearrangements by repertoire analysis was compared with that by

use of the six V family-specific framework 1 primers and the consensus J primer previously published[7] by the BIOMED-2 consortium. Each amplification reaction used 100 ng of genomic DNA. The end point was the ability to detect complete *IGH* rearrangements.

Conventional MRD Detection Methodologies Used for the Collaborative Study

In the second study, the *IGH* repertoire was assessed in 50 patients and compared with MRD markers previously identified by the Sydney laboratory. These MRD tests were performed according to the methods and guidelines developed by the BFM MRD task force and the European Study Group on Minimal Residual Disease in Acute Lymphoblastic Leukemia (ESG-MRD-ALL).[11,12] RQ-PCR quantification was performed for rearrangements of immunoglobulin heavy and κ genes (*IGH, IGK*) and T-cell receptor δ, δ-α, β, and γ genes (*TCRD, TCRD-A, TCRB* and *TCRG*). The identification of mature and immature markers was based on 10 PCR reactions using primers previously published for VDJ and DJ *IGH* rearrangements.[13] The family-specific primers for VH1/7, VH2, VH3, VH4, VH5, VH6, DH2, DH3, and DH6 were tested individually with a common J primer, and a multiplex PCR was used for the rarer DH1, DH4, DH5 and DH7 with the same J primer. For identification of other Ig/TCR markers, family-specific primers were used in singleplex reactions for *TCRG*, *TCRD*,[6] and in multiplex PCRs for *TCRB* and *TCRD-A.*[7,14] The PCR reactions for five patients for all markers except were performed simultaneously in two 96-well plates with appropriate positive and negative controls. Following PCR, the DNA was heteroduplexed and analyzed on polyacrylamide gels to detect clonal rearrangements[15] that were then verified by sequencing.

Results

Initial Study

The number of *IGH* rearrangements detected in each patient using genomic DNA is shown in Table 2. The median number of complete *IGH* rearrangements detected per patient was two in children and one in adults ($P = 0.016$ for age difference, Mann-Whitney test, two-tailed). Rearrangement was not detected in one child and one adult. One rearrangement marking the major clone was detected in 11 children and 13 adults, and two such rearrangements were detected in 13 children and four adults. Repertoire analysis thus detected one or more markers for the major clone in 96% of children and 94% of adults.

Table 2. Number of Rearrangements Detected Using Genomic DNA in Each Patient in the First Study of 25 Children and 18 Adults

Type of rearrangement	None detected	VDJ					DJ	
Number of rearrangements	0	1	2	3	4	5	1	2
Childhood ALL	1	2	10	8	0	1	2	1
Adult ALL	1	11	5	0	0	0	0	1

Table 3. Number of Rearrangements of High (100–10%), Intermediate (10–1%), or Low (<1%) Abundance Detected by the Repertoire and/or BIOMED-2 Approaches in the First Study

Relative abundance of rearrangement	100–10%	10–1%	<1%
Detected by both analyses	29	2	6
BIOMED-2 only	1	0	1
Repertoire analysis only	7	1	23

Repertoire analysis was performed using genomic DNA in 15 children and using pre-amplified DNA in 10. The BIOMED-2 analysis was performed in all 25 using genomic DNA. Rearrangements were grouped by their abundance, assessed as the percentage of the total number of rearranged *IGH* molecules that each rearrangement contributed in a patient.

Repertoire analysis on preamplified DNA, also performed in 10 children and 10 adults, detected all previously detected rearrangements, but also detected additional 0 to five rearrangements per patient in both children and adults. These additional rearrangements, 23 in children and 17 in adults, were presumed to mark small minor leukemic clones. Sequencing revealed that, for children, 10 of the 23 minor clones detected were unrelated to the major clone, whereas, for adults, only one of the 17 minor clones detected were unrelated. In two children the rearrangement predominating at diagnosis appeared to have been derived from a founder rearrangement, which was also identified, but at a level of <0.1%.

The comparison of repertoire analysis and the BIOMED-2 method for detection of complete *IGH* rearrangements is shown in Table 3. The repertoire approach detected a greater number of both high-abundance rearrangements ($P = 0.03$, Fisher's exact test, one-tailed) and low abundance rearrangements ($P = 0.001$, Fishers exact test, one-tailed) than did the BIOMED-2 approach. The BIOMED-2 approach did not detect seven high-abundance rearrangements owing to concomitant amplification of two rearrangements, which resulted in superimposed and unreadable sequences, and it did not detect rearrangements of intermediate or low abundance. The two rearrangements detected only by the BIOMED-2 approach both involved a pseudogene, which the repertoire analysis primers were not designed to detect.

Collaborative Study

The repertoire of *IGH* rearrangements was determined for 50 clinical trial patients for whom the conventional ESG-MRD-ALL method had been previously used to identify sensitive markers for MRD-based patient risk stratification. The conventional method using family-specific primers identified 206 markers in these patients including 86 *IGH* rearrangements and 120 rearrangements for *IGK, TCRG, TCRD, TCRD-A*, and *TCRB*. *R*epertoire analysis identified 154 *IGH* rearrangements.

The numbers of *IGH* rearrangements detected by the two methods are shown in more detail in Table 4. The

Table 4. Number of Rearrangements Detected in 50 Children by the Conventional Method Using Family-Specific Primers and by Repertoire Analysis Using Segment-Specific Primers

Clones marked by rearrangement	Major clone	Minor clones			
Relative abundance of rearrangement	100–10%	>10%	10–1%	1−0.1%	<0.1%
Detected by both analyses	61	8	6	1	4
Conventional only	4	1	0	1	0
Repertoire analysis only	23	5	12	10	24
Probability	<0.0001	NS	<0.0001	<0.001	<0.0001

The criteria for major and minor clones are described in Materials and Methods. Rearrangements have been grouped by abundance and the probabilities that the differences in detection of rearrangements arose by chance are shown. NS, not significant.

numbers of complete VDJ and incomplete DJ rearrangements have been pooled and rearrangements have been classified according to whether they mark the major or minor clones and on their abundance. *IGH* repertoire analysis detected significantly more rearrangements marking both major and minor clones ($P < 0.001–0.0001$, Fisher's exact test, one-tailed). Factors that appeared to contribute to the difference between the two approaches in the number of *IGH* rearrangements detected include: for the ESG-MRD-ALL approach, not isolating and sequencing faint PCR products in some cases and not individually sequencing biclonal *IGH* PCR products unless there were no alternate markers, and for repertoire analysis, the preamplification step that provided sufficient DNA to enable repertoire analysis to then detect rearrangements of intermediate and low abundance.

When markers for the major clone are considered, both laboratories detected one or more such markers in 48 patients of the 50 patients (96%). The Sydney laboratory found two sensitive rearrangements suitable for patient stratification in 39 patients, one in nine patients, and none in two patients. Since *IGH* rearrangements enable very sensitive monitoring of MRD by nested PCR, down to 10^{-6},[16] we regarded an *IGH* rearrangement detected by repertoire analysis and marking the major clone as being a marker suitable for sensitive quantification of that clone. Using this criterion, the Flinders laboratory found two such markers in 36 patients, one in 12 patients, and none in two patients. If the results from all of the 75 children studied by repertoire analysis at Flinders are pooled, at least one sensitive marker for the major clone was detected in 96% of children, and two such markers were detected in 65%. For the 50 patients studied by both laboratories, there were 86 *IGH* markers detected by the Sydney laboratory, but 41 were not used for MRD monitoring because other markers, which had better quantitative ranges or sensitivity in the standard MRD RQ-PCR tests, were used instead. Thus *IGH* markers comprised 45 of the 87 markers actually used to measure MRD in the Sydney laboratory but all of the 84 potentially usable markers detected by the Flinders laboratory.

With regard to minor clones, repertoire analysis detected 0 to six clones per patient, the mean number per patient being 1.40. Most were small and ranged in size down to approximately 0.01%, but in 12 patients there was a total of 13 minor clones, which each comprised over 15% of the leukemic population. Cytogenetic information was not available on these patients, and it was not known if any had trisomy 14. Of the 70 minor clones identified, sequence analysis showed that 21 had a lineage relationship to the major clone, whereas 49 did not. In two patients, a small minor clone appeared to be the ancestor of the major clone present at diagnosis.

To investigate whether the preamplification step might improve detection of minor clones by the BIOMED-2 primers, three samples in which a number of minor clones had been identified were amplified to the same extent, either by the BIOMED-2 primers alone or by the BIOMED-2 primers following a preliminary preamplification. Electrophoresis of the amplified products showed no differences.

Discussion

This investigation of the *IGH* repertoire approach in ALL has passed through three phases. The first phase involved development of the methodology and study of the *IGH* repertoire using genomic DNA. The results indicated that at least one abundant *IGH* rearrangement could be detected in the great majority of both children and adults. More than two rearrangements were seen in some children indicating the presence of minor leukemic clones.

The second phase involved a preliminary amplification of all *IGH* rearrangements, which enabled detection of additional rearrangements marking small leukemic clones. When detection of *IGH* rearrangements using repertoire analysis was compared with detection of *IGH* rearrangements using the panel of primers for this purpose suggested by BIOMED-2, repertoire analysis detected significantly more *IGH* rearrangements marking both the major and minor leukemic clones.

The third phase involved a comparison, using the same patient samples, between repertoire analysis, performed by the Flinders laboratory, and the conventional strategy, performed by the Sydney laboratory using the same methods and MRD stratification system as are used for the large AIEOP-BFM ALL 2000 cohort.[11,12,17] Three conclusions emerged. First, both methods were suitable for detecting one or two markers for the major leukemic clone; at least one marker was detected in 96% of patients by both approaches and at least two markers were detected in 72% of patients by repertoire analysis and 78% of patients by the conventional approach. Second, the repertoire approach detected significantly more *IGH* markers for the major leukemic clone. Third, repertoire

analysis detected many more *IGH* markers for minor leukemic clones.

Our results suggest that the enhanced ability of repertoire analysis to detect *IGH* markers is largely or wholly attributable to the use of segment-specific rather than family-specific primers. The preamplification step was introduced to provide sufficient DNA to enable repertoire analysis to be performed subsequently. It seems unlikely that preamplification would materially improve detection of minor rearrangements by family-specific primers, since in most cases their amplification in a PCR would be limited owing to concomitant amplification of nonspecific material or of one or more other rearrangements of higher abundance. An experiment to directly investigate whether preamplification improved detection of minor clones by the BIOMED-2 primers gave a negative result and further experimentation on this point was not pursued.

The enhanced ability of repertoire analysis to detect *IGH* rearrangements marking the major clone may be an advantage for detection and quantification of MRD. Owing to their structure, with two N regions and often a substantial D region, *IGH* rearrangements usually enable the design of several primers of relatively high specificity. In parallel studies, in 23 of the 25 children who were investigated in the initial phase of this study, *IGH* primers derived as the result of repertoire analysis together with nested PCR enabled MRD to be detected down to approximately 10^{-6}.[16] As discussed in more detail in that paper, the ability to quantify the major leukemic clone present at diagnosis down to a very low level during treatment may improve precision of quantification of MRD for patients with MRD in the range of 10^{-3} to 10^{-4} and should improve both detection and quantification of MRD for patients with a level in the range of 10^{-4} to 10^{-6}. Since many or most children with ALL have an MRD level at the end of induction of $<10^{-4}$, improved quantification of MRD using an *IGH* marker identified by repertoire analysis has the potential to improve clinical decisions on treatment in many patients.

The ability of repertoire analysis to detect *IGH* rearrangements marking minor leukemic clones may also prove to have clinical utility. In a substantial minority of patients who relapse, the relapse clone carries a marker rearrangement that was not detected at diagnosis.[18–21] However, retrospective studies using this marker sequence for the relapse clone have suggested that this clone is often present at diagnosis but not detected owing to its size,[22,23] and several studies have indicated that it is already chemoresistant at that time.[24,25] Since *IGH* repertoire analysis can identify clone-specific markers for minor leukemic clones at diagnosis, its use, in conjunction with sensitive nested PCR, might well enable chemoresistant minor clones to be prospectively identified, which in turn may improve prediction of outcome in childhood ALL. For identification of chemoresistant minor clones related to the major clone, a clone-specific marker identified by repertoire analysis is likely to be more useful than a marker that is common to both the major and the minor clone, since changes in the size of the major clone may obscure changes in size of the minor clone. The resources required for identification of chemoresistant minor clones might be lessened by using multiplex PCR and monitoring the response of only the relatively large minor clones, such as those comprising >1% of the leukemic population.

Apart from their implications for measurement of MRD, the results of *IGH* repertoire analysis also provide information on the origin and evolution of leukemic clones in ALL. Minor clones were observed in 67% of the children and 50% of the adults in whom repertoire analysis was performed using preamplified material. Most of the minor clones were small but in 12 of the 50 patients in the second study there was a rearrangement that marked a minor clone comprising >15% of the leukemic population. In two instances in each study a small minor clone appeared to be the ancestor of the major clone present at diagnosis. A lineage unrelated to that of the major clone was seen in 59 of the 93 minor clones detected in children from both studies but in only one of the 17 minor clones detected in adults. These observations on lineage relationships present at diagnosis resemble the observations on lineages observed in children and adults at the time of relapse, and support the concept that clonal evolution in leukemia is usually due to selection of clones present at diagnosis rather than the emergence of completely new clones. The observed difference between children and adults in the proportion of minor clones that are unrelated to the major clone could be explained if leukemia develops some years before clinical presentation and before gene rearrangement in some children.

Repertoire analysis may also prove to have utility in lymphoid neoplasms other than ALL, both for understanding clonal biology and for identification or monitoring of the leukemic clone. For example, in lymphoma it may be difficult to identify the presence of a clonal population of lymphocytes owing to the concomitant presence of many non-leukemic lymphocytes, and determination of the clonal repertoire in biopsy or aspiration specimens may help to overcome this problem.

Finally, although our results suggest that *IGH* repertoire analysis has potential advantages over the current standard approach, it probably consumes more time and resources, although not prohibitively more. Many of the PCRs are performed using stored microplates into which primers have previously been robotically aliquoted and dried down. Preliminary testing of primers for sensitivity and specificity does not seem to be necessary. The steps of marker identification, sequencing and primer synthesis can be performed sufficiently rapidly to enable timely MRD measurement. Treatment decisions based on MRD level may have substantial personal and resource implications, and any increased use of resources resulting from marker detection using repertoire analysis must be balanced against potential patient benefits and resource savings resulting from improvement in MRD measurement as a consequence of using IgH markers.

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